

IPSE/alpha-1, a major secretory glycoprotein antigen from schistosome eggs, expresses the Lewis X motif on core-difucosylated N-glycans

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Schistosomes are parasitic flatworms that infect millions of people in (sub)tropical areas around the world. Glycoconjugates of schistosomes play a critical role in the interaction of the different developmental stages of the parasite with the host. In particular, glycosylated components of the eggs produced by the adult worm pairs living in the bloodstream are strongly immunogenic. We have investigated the glycosylation of interleukin-4-inducing factor from schistosome eggs (IPSE/alpha-1), a major secretory egg antigen from *Schistosoma mansoni* that triggers interleukin-4 production in human basophils, by MS analysis of tryptic glycopeptides. Nanoscale LC-MS(/MS) and MALDI-TOF(/TOF)-MS studies combined with enzymatic degradations showed that monomeric IPSE/alpha-1 contains two N-glycosylation sites, which are each occupied for a large proportion with core-difucosylated diantennary glycans that carry one or more Lewis X motifs. Lewis X has been reported as a major immunogenic glycan element of schistosomes. This is the first report both on the expression of Lewis X on a specific schistosome egg protein and on a protein-specific glycosylation analysis of schistosome eggs.

Schistosomiasis is a chronic parasitic infection, with more than 200 million people affected worldwide [1]. *Schistosoma mansoni*, one of the major schistosome species, may persist in human blood vessels for over a decade if unchallenged by drug treatment. Each pair of male and female worms produces hundreds of eggs per day that play a major role in the morbidity associated with schistosomiasis. It has become clear that glycosylated molecules produced by schistosome eggs are strongly immunologically active, both in the human host as well as in the mouse infection model.

Glycan epitopes of egg glycolipids and glycoproteins, shared in many cases with adult and larval stages of the schistosome, are the predominant targets of the humoral immune response in schistosomiasis [2–4]. Elaborate structural studies have shown that egg-associated glycans contain often fucosylated structural elements, such as Gal(β1-4)[Fuc(α1-3)]GlcNAc (Lewis X), GalNAc(β1-4)[Fuc(α1-3)]GlcNAc (LDN-F), Fuc(α1-3)GalNAc(β1-4)GlcNAc (F-LDN), and Fuc(α1-2)Fuc(α1-3)GalNAc(β1-4)[Fuc(α1-2)Fuc(α1-3)]GlcNAc (DF-LDN-DF) [5–11]. The antibody responses against Lewis X, LDN-F, F-LDN(-F) and (DF-)LDN-DF have

Abbreviations

F, deoxyhexose; F-LDN(-F), Fuc(α1-3)GalNAc[±Fuc(α1-3)](β1-4)GlcNAc; H, hexose; IL, interleukin; IPSE/alpha-1, interleukin-4-inducing factor from schistosome eggs; LacNAc, Gal(β1-4)GlcNAc; LDN, LacdiNAc, GalNAc(β1-4)GlcNAc; LDN-F, GalNAc(β1-4)[Fuc(α1-3)]GlcNAc; Lewis X, Gal(β1-4)[Fuc(α1-3)]GlcNAc; N, N-acetylhexosamine; PBMC, peripheral blood mononuclear cell; SEA, schistosome egg antigen; Th1, T helper 1; Th2, T helper 2.

been studied in detail, revealing that in particular Fuc(α 1-3)GalNAc β 1- and Fuc(α 1-2)Fuc α 1-containing glycans are major targets of the humoral immune response in schistosomiasis [4,12–14]. Most of these structural elements have been detected on egg glycoproteins, as well as glycolipids, by defined mAbs [5]. The Lewis X determinant, however, seems to be mainly expressed on egg proteins, while it is present only in trace amounts on egg glycolipids [5]. Structural studies on N-glycans released from an overall mixture of *S. mansoni* egg glycoproteins indicated, in addition, that egg N-glycans are based on xylosylated (α 1-6)-fucosylated as well as (α 1-3)/(α 1-6)-difucosylated N-glycan core structures [15].

It has been established that egg components are responsible for the strong polarization of the host's immune response from a T helper 1 (Th1) to a T helper 2 (Th2) type [16] after the onset of egg production, which signals the start of the chronic stage of the disease [17]. Th2-type responses mediated by lymphocytes in a murine model of intranasal sensitization were found to be driven by schistosome egg antigens (SEAs) with intact carbohydrate moieties, but not with metaperiodate-treated egg antigens [18]. In particular, the immunomodulatory effects of the Lewis X antigen, which has initially been shown to be capable of inducing the production of mediators such as interleukin (IL)-10 and prostaglandin E₂, associated with Th2 responses in infected mice, have been studied in detail [19]. However, additional glycan elements, such as LDN-DF, present on schistosome egg glycoproteins may also be active as inducers of pro- or anti-inflammatory cytokines associated with schistosomiasis [20,21].

Surprisingly, despite the strong interest in schistosome egg-derived glycans as immunomodulators, and the various studies into the immunological/biological activities of individual egg glycoproteins [22–24], not a single study on the glycosylation of any egg protein has been reported to date. One of the major secretory glycoproteins of *S. mansoni* is the IL-4-inducing factor from schistosome eggs (IPSE, also known as α -1) [24–27], which triggers IL-4 release from basophils [24]. To determine which glycans are expressed by IPSE/ α -1, we performed a detailed MS-based structural study. It was found that Lewis X is the major terminal glycan motif in the antennae of N-glycans on IPSE/ α -1, in combination with α 1-3- and/or α 1-6-fucosylation of the chitobiose core. The pronounced difference between the overall egg N-glycosylation profile [16] and the specific N-glycan pattern found on IPSE/ α -1, underlines the importance of the protein-specific analysis of *S. mansoni* egg glycosylation.

Results

Mass of intact IPSE/ α -1

The primary sequence of IPSE/ α -1 (Fig. 1), together with its biological activity as an IL-4-inducing factor, have been published previously [24]. In the current study, the possible post-translational modifications, in particular glycosylation, have been analysed by a combination of mass spectrometric and chromatographic techniques. By MALDI-TOF-MS, intact IPSE/ α -1 was detected as a broad peak at $\approx$ 35 kDa, with a shoulder at $\approx$ 33 kDa, which was interpreted as a heterogeneous mixture of different glycoforms (Fig. 2A,B; discussed below). In a double-protonated form, it gave a signal at $\approx m/z$ 17 000. IPSE/ α -1 is known to be a disulfide-linked homodimer of identical protein subunits, with the calculated peptide mass of the monomer being 13.2 kDa [24]. Thus, the observed mass of 33–35 kDa indicates that the dimer would carry modifications of a mass of $\approx$ 8.5 kDa. These modifications were expected to be mainly glycans, as earlier findings indicated that IPSE/ α -1 is a glycoprotein [24].

Site-specific N-glycosylation of IPSE/ α -1

For analysis of its glycosylation, IPSE/ α -1 was cleaved with trypsin, and the resulting (glyco-)peptides were analyzed by MALDI-TOF-MS and nano-LC-MS/MS. Glycopeptides were analyzed either intact or after treatment with PNGase A and β -galactosidase. In MALDI-TOF-MS of the total tryptic digest, two glycopeptide clusters were registered in the high mass



Fig. 1. Primary amino-acid sequence of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1). Cysteines (circles) and N-glycosylation sites (boxes) were labeled. Disulfide bridges are indicated by dotted lines. The sequence covered by the LC-MS/MS data is underlined. The signal peptide M1–S20 is absent from the purified native IPSE/ α -1 and is shown in lower-case text. Bold type, expected tryptic cleavage site.

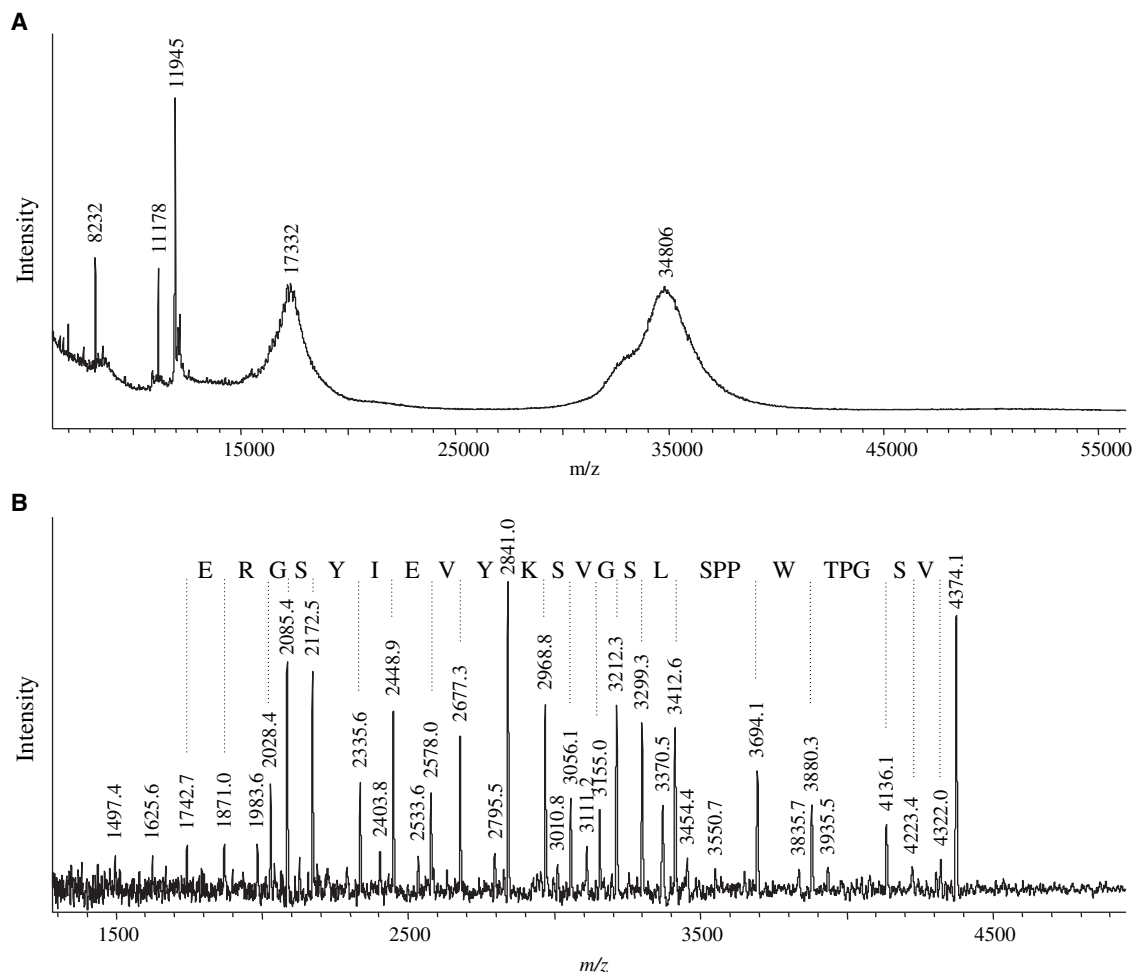


Fig. 2. MALDI-TOF-MS of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1). (A) Broad signals were registered at ≈ 34 kDa (singly charged) and ≈ 17 kDa (doubly charged) in the linear positive-ion mode. (B) In the low-mass region, an extensive series of Y-ions was detected arising from in-source decay.

range (Fig. 3). Glycopeptides differed in the content of hexoses (H), *N*-acetylhexosamines (N), and deoxyhexoses (F), as reflected by corresponding mass differences (162 Da, 203 Da and 146 Da, respectively). The glycopeptide patterns of the two clusters were very similar, which becomes obvious by the aligned presentation chosen in Fig. 3. For cluster one (Fig. 3A), the assumption of an E69–R84 peptide moiety substituted with an N-glycan at the consensus N-glycosylation site, N80, resulted, for the two major glycopeptide species (m/z 4009 and 4155), in the deduced glycan composition $H_5N_4F_2$ and $H_5N_4F_3$. Assuming a similar glycan composition of $H_5N_4F_2$ and $H_5N_4F_3$ for the aligned major glycopeptides of cluster 2 at m/z 5867 and 6013 (Fig. 3B), the deduced mass of the peptide moiety fitted with the two disulfide-linked peptides S45–K67 and I85–K99, the former contain-

ing the consensus N-glycosylation site, N64. This tentative assignment of peptide and glycan moieties was confirmed by glycopeptide fragmentation analysis and enzymatic treatment, as shown below. Registered masses, as well as deduced glycan compositions for all the detected glycopeptide species, are given in Table 1.

In order to analyse the two glycosylation sites independently, we performed an RP-HPLC fractionation of the total tryptic (glyco-)peptides. Fractions of 0.5 mL were collected and analysed by MALDI-TOF-MS. Tryptic peptides covering the N-glycosylation site, N80, were found back in fractions 41–43. Whilst glycopeptides with the largest glycan moieties were found in fraction 41, small glycan moieties resulted in enhanced retention (fraction 43). Fractions 41 (see Table 1) and 42 (Fig. 4 and Table 1) were subjected

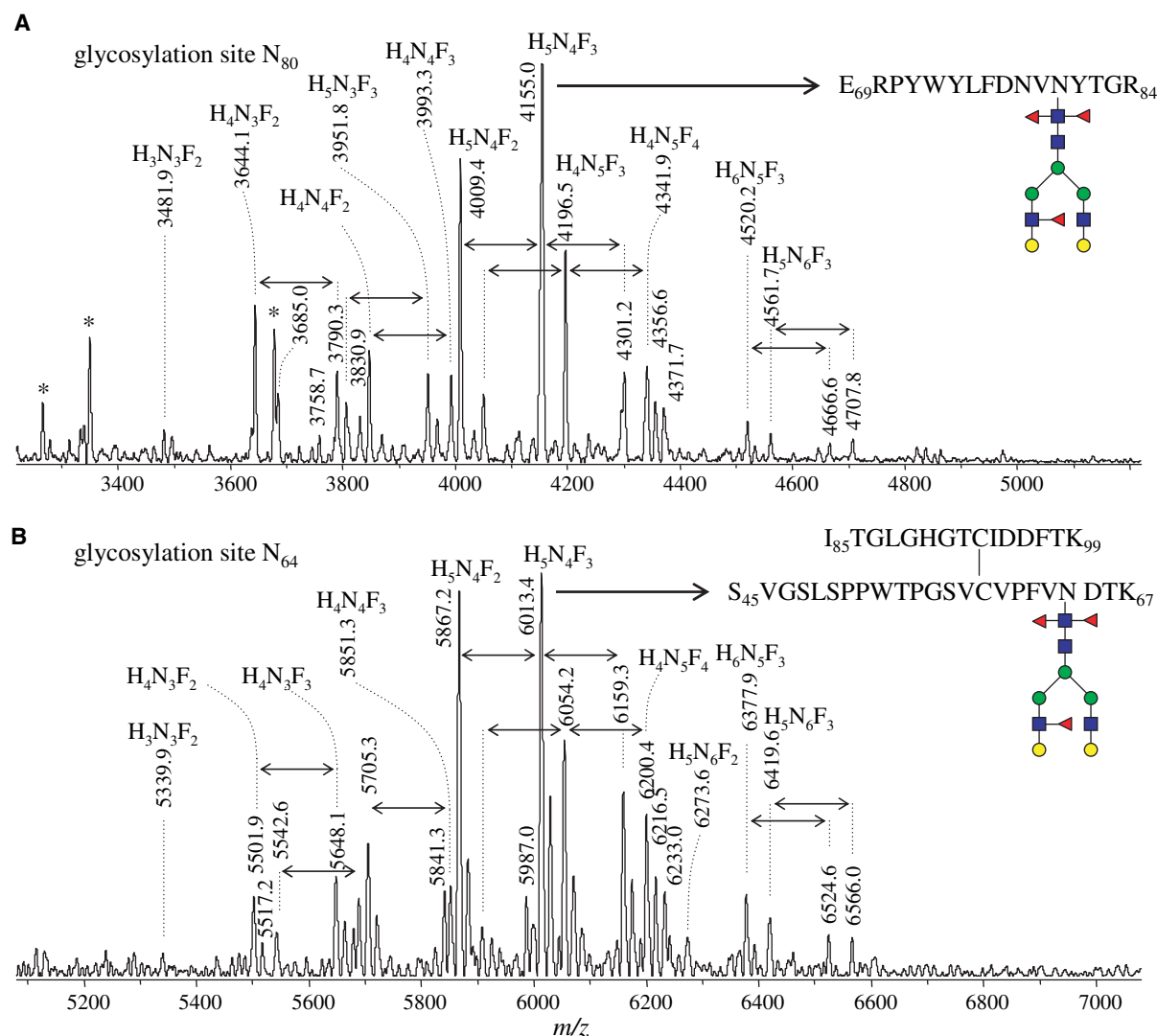


Fig. 3. MALDI-TOF-MS of glycopeptides from a tryptic digest of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1). The tryptic glycopeptides covering the glycosylation site, N80 (A), and the tryptic, disulfide-linked dipeptides containing glycosylation site N64 (B), were analysed in the positive-ion reflectron mode. Signals are labeled with average masses. Compositions of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N), and fucose (F). Differences in fucose content are indicated by double-headed arrows. Non-glycopeptide signals are marked with asterisks (*). Red triangle, fucose; yellow circle, galactose; blue square, *N*-acetylglucosamine; green circle, mannose.

to β -galactosidase treatment, which resulted in the loss of two, one or no hexose residues from individual glycopeptide species. The loss of two hexoses indicated N-glycan H₅N₄F₂ to contain two nonfucosylated LacNAc antennae, which are both sensitive to β -galactosidase treatment (the peak at m/z 4010 in Fig. 4A shifts to m/z 3685 in Fig. 4B). The two fucoses of this N-glycan would therefore be located at the core. Similar information was obtained for most of the other complex-type N-glycans of site N80, as summarized in Table 1.

A population of glycopeptides detected in RP-HPLC fractions 23–25 (Fig. 5) exhibited a glycan profile similar to the profiles shown in Fig. 3 for the tryptic peptide comprising the two N-glycosylation sites. In order to identify the peptide moiety, the glycopeptide at m/z 3261 in fraction 24 was subjected to MALDI-TOF/TOF-MS (data not shown). A characteristic pattern of signals at m/z 1180 (protonated peptide NH₃), m/z 1198 (protonated peptide), m/z 1281 (^{0,2} X-ring fragmentation of the innermost *N*-acetylglucosamine), m/z 1547 (protonated peptide with *N*-acetylglucosamine

Table 1. Interleukin-4-inducing factor from schistosome eggs (IPSE/alpha-1) glycopeptide species detected after trypsin digestion. IPSE/alpha-1 was trypsinized and resulting glycopeptides were analyzed by MALDI-TOF-MS and nano-LC-MS/MS. Glycan compositions are given in terms of hexose (H), N-acetylhexosamine (N), and fucose (F). For the glycans detected on IPSE/alpha-1, a compositional comparison is performed with the glycans described previously [15] by sequential glycan release from *Schistosoma mansoni* eggs with PNGase F (PF) and PNGase A (PA): X, not detected by the earlier study [15]; v, detected at a significant level in at least one preparation; O, consistently present as the major signals. Average masses of glycopeptide precursors are given throughout. Glycopeptides were registered by MALDI-TOF-MS as $[M+H]^+$. For glycopeptides registered by nano-LC-MS, charge states are specified. Peptide moieties were tryptic as indicated in Fig. 4. For many glycopeptides, masses registered by MALDI-TOF-MS after β -galactosidase treatment are listed (β -gal), and the loss of n hexose is concluded (– n hex). Characteristic fragment ions are given as registered by nano-LC-MS/MS. 3,6F (α 1–3/(α 1–6)-di-fucosylation of the innermost GlcNAc).

Glycan composition	Release		Glycosylation site	Glycopeptide signal (m/z)	Fragment ions	Structure characteristics	Structure
	PF	PA					
H ₂ N ₂ F ₁	v	X	N ₈₀	2076.6 ^a	–	–	–
H ₂ N ₂ F ₂	X	X	N ₈₀	2222.5 ^a , 1110.7 ^a [M+2H] ²⁺	–	–	–
H ₃ N ₂ F ₁	v	X	N ₈₀	2238.5 ^a , 1118.7 ^a [M+2H] ²⁺	–	–	–
H ₃ N ₂ F ₂	X	X	N ₈₀	3279.5; 2384.8 ^a 1191.5 ^a [M+2H] ²⁺	see Fig. 7	trimannosyl core, 3,6F	N9
H ₄ N ₂ F ₂	X	X	N ₈₀	2547.0 ^a , 1272.5 ^a [M+2H] ²⁺	852 (H ₄ N ₁); 1693 (pep-N ₁ F ₂)	3,6F	3,6F
H ₃ N ₃ F ₂	X	X	N ₈₀	3481.9; 2588.1 ^a , 1293.0 ^a [M+2H] ²⁺	1693 (pep-N ₁ F ₂)	3,6F	3,6F
H ₄ N ₃ F ₂	X	v	N ₆₄	5339.9; 1336.2 [M+4H] ⁴⁺	–	–	–
			N ₈₀	3644.1 (β -Gal: 3481.9; –1 Hex); 2750.3 ^a ; 916.6 ^a [M+3H] ³⁺ ; 1373.8 ^a [M+2H] ²⁺	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 847 (pep-N ₁ F ₂); 1693 (pep-N ₁ F ₂)	1 LacNAc, 3,6F	N3
H ₃ N ₄ F ₂	X	X	N ₆₄	5501.9; 1100 [M+5H] ⁵⁺ ; 1374.8 [M+4H] ⁴⁺	–	–	–
H ₄ N ₃ F ₃	X	X	N ₈₀	3685.0; 2791.1 ^a ; 930.3 ^a [M+3H] ³⁺ ; 1394.5 ^a [M+2H] ²⁺	407 (N ₂); 847 (pep-N ₁ F ₂)	1 LacdiNAc, 3,6F	N5
			N ₆₄	5542.6; 1384.8 [M+4H] ⁴⁺	–	–	–
			N ₈₀	3790.3; (β -Gal: 3790.2; –0 Hex); 2896.2 ^a ; 965.2 ^a [M+3H] ³⁺ ; 1446.9 ^a [M+2H] ²⁺	512 (H ₁ N ₁ F ₁); 658 (H ₁ N ₁ F ₂); 1693 (pep-N ₁ F ₂)	1 Lewis X, 3,6F	N3
H ₅ N ₃ F ₂	v	X	N ₆₄	5648.1; 1129.8 [M+5H] ⁵⁺ ; 1411.1 [M+4H] ⁴⁺	–	–	–
			N ₈₀	3804.9 (β -Gal: 3805.9; –0 Hex); 2912.5 ^a ; 970.5 ^a [M+3H] ³⁺ ; 1455.2 [M+2H] ²⁺	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 847 (pep-N ₁ F ₂)	1 Lewis X	N6
H ₃ N ₄ F ₃	X	X	N ₆₄	5663.7; 1384.7b [M+3H] ³⁺	–	–	–
			N ₈₀	3830.9; 2937.3 ^a ; 979.5 ^a [M+3H] ³⁺ ; 1467.6 ^a [M+2H] ²⁺	–	–	–
			N ₆₄	5689.0	–	–	–
H ₄ N ₄ F ₂	v	X	N ₈₀	3847.1 (β -Gal: 3684.9; –1 Hex); 2953.4 ^a ; 984.4 ^a [M+3H] ³⁺ ; 1475.5 ^a [M+2H] ²⁺	366 (H ₁ N ₁); 407 (N ₂); 847 (pep-N ₁ F ₂)	1LacNAc, 1 single GlcNAc, 3,6F	N4
H ₃ N ₅ F ₂	X	X	N ₆₄	5705.3; 1141.2 [M+5H] ⁵⁺ ; 1425.7 [M+4H] ⁴⁺	366 (H ₁ N ₁);	1 LacNAc	–
H ₅ N ₃ F ₃	X	X	N ₈₀	2994.5 ^a , 998.1 ^a [M+3H] ³⁺ ; 1497.1 ^a [M+2H] ²⁺	–	–	–
			N ₆₀	3951.8 (β -Gal: 3951.7; –0 Hex); 3058.6 ^a ; 1019.5 ^a [M+3H] ³⁺	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁)	1 Lewis X, 3,6F	N6
H ₆ N ₃ F ₂	X	X	N ₈₀	3969.0 (β -Gal: 3805.9; –1 Hex); 3074.9 ^a ; 1025.1 ^a [M+3H] ³⁺	366 (H ₁ N ₁)	1LacNAc, 3,6F	–
H ₄ N ₄ F ₃	X	X	N ₈₀	3993.3 (β -Gal: 3993.0; –0 Hex); 3099.8 ^a ; 1032.9 ^a [M+3H] ³⁺	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 1693 (pep-N ₁ F ₂)	1 Lewis X, 1 single GlcNAc, 3,6F	N4
H ₅ N ₄ F ₂	v	O	N ₆₄	5851.3	–	–	–
			N ₈₀	4009.4 (β -Gal: 3684.9; –2 Hex); 3115.4 ^a ; 1038.4 ^a [M+3H] ³⁺ ; 5867.2; 1466.3 [M+4H] ⁴⁺	366 (H ₁ N ₁); 847 (pep-N ₁ F ₂)	2 LacNAc, 3,6F LacNAc	N1
H ₃ N ₅ F ₃	X	X	N ₈₀	3140.4 ^a	366 (H ₁ N ₁)	–	–

Table 1. (Continued).

Glycan composition	Release		Glycosylation site	Glycopeptide signal (<i>m/z</i>)	Fragment ions	Structure characteristics	Structure
	PF	PA					
H ₄ N ₅ F ₂	X	X	N ₈₀	4050.0 (β -Gal: 3887.6; -1 Hex); 3156.5 ^a , 1052.0 ^a [M+3H] ³⁺ 5908.2; 1180 1476.5 [M+4H] ⁴⁺ 3220.5 ^a	366 (H ₁ N ₁); 407 (N ₂); 512 (H ₁ N ₁ F ₁); 553 (N ₂ F ₁)	1 LacNAc, 1 fucosylated LacdiNAc	N2
H ₆ N ₄ F ₃	X	X	N ₆₄		–	–	–
H ₆ N ₄ F ₃	v	O	N ₈₀	4155.0 (β -Gal: 3993.3; -1 Hex); 3261.6 ^a , 1087.2 ^a [M+3H] ³⁺ 6013.4; 1202.7 [M+5H] ⁵⁺ ; 1502.8 [M+4H] ⁴⁺ ; 1131.6 ^a [M+3H+ Na] ⁴⁺	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 847 (pep-N ₁ F ₂) see Fig. 10	1 Lewis X, 1 LacNAc, ^{3,6} F	N1
H ₄ N ₅ F ₃	X	X	N ₈₀	4196.5 (β -Gal: 4199.1; -0 Hex, and 4034.2; -1 Hex); 3302.9 ^a , 1100.9 ^a [M+3H] ³⁺ 6054.2; 1210.8 [M+5H] ⁵⁺ ; 1512.8 [M+4H] ⁴⁺ 4301.2 (β -Gal: 4301.2; -0 Hex); 1075 [M+4H] ⁴⁺ ; 3408.0 ^a , 1136.0 ^a [M+3H] ³⁺ 6159.3; 1232.1 [M+5H] ⁵⁺ ; 1539.1 [M+4H] ⁴⁺ 1167.7 ^b [M+3H+ Na] ⁴⁺	see Fig. 9 366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 407 (N ₂) see Fig. 8 512 (H ₁ N ₁ F ₁)	1 LacNAc/Lewis X, 1 (fucosylated) LDN, ^{3,6} F	N2
H ₅ N ₄ F ₄	X	v	N ₆₄		–	–	–
H ₄ N ₅ F ₄	X	X	N ₈₀	4341.9 (β -Gal: 4342.4; -0 Hex); 3449.1 ^a , 1149.8 ^a [M+3H] ³⁺ 6200.4; 1239.9 [M+5H] ⁵⁺ ; 1549.0 [M+4H] ⁴⁺ 1177.9 ^b [M+3H+ Na] ⁴⁺ 1562.9 ^b [M+3H] ³⁺ 3465.1 ^a 6216.5 3481.0 ^a 6233.0 3522.2 ^a 6273.6	512 (H ₁ N ₁ F ₁); 553 (N ₂ F ₁); 658 (H ₁ N ₁ F ₂); 699 (N ₂ F ₂); 847 (pep-N ₁ F ₂) – – – – – –	1 Lewis X, 1 fucosylated LDN, ^{3,6} F	N2
H ₅ N ₅ F ₃	X	X	N ₆₄		–	–	–
H ₆ N ₅ F ₂	X	X	N ₈₀		–	–	–
H ₅ N ₆ F ₂	X	X	N ₈₀		–	–	–
H ₆ N ₅ F ₃	X	X	N ₆₄	4520.2 (β -Gal: 4197.0; -2 Hex); 3627.2 ^a , 1208.9 [M+3H] ³⁺ 6377.9	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁);	LacNAc, Lewis X	N7
H ₅ N ₆ F ₃	X	X	N ₈₀	4561.7 (β -Gal: 4399.9; -1 Hex); 1140; 3668.3 ^a , 1222.5 ^a [M+3H] ³⁺ 917.0 ^a [M+4H] ⁴⁺ 6419.6; 1283.9 [M+5H] ⁵⁺ 3709.5 ^a	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 407 (N ₂); 553 (N ₂ F ₁) – –	1 LacNAc, 1 Lewis X, 1 (fucosylated) LDN	N8
H ₄ N ₇ F ₃	X	X	N ₆₄		–	–	–
H ₆ N ₅ F ₄			N ₈₀	4666.6 (β -Gal: 4668.2.4; -0 Hex, 4505.9; -1Hex); 3773.4 ^a ; 1257.1 ^a [M+3H] ³⁺ 6524.6	366 (H ₁ N ₁); 512 (H ₁ N ₁ F ₁); 658 (H ₁ N ₁ F ₂); 847 (pep-N ₁ F ₂) –	2 LacNAc, 1 Lewis X, ^{3,6} F; or 1 LacNAc, 2 Lewis X	N7
H ₅ N ₆ F ₄	X	X	N ₆₄		–	–	–
H ₆ N ₇ F ₄	X	X	N ₈₀	4707.8 (β -Gal: 4708.5; -0 Hex); 3814.5 ^a 6566.0 3855.4 ^a	– – –	– – –	N8

^aGlycopeptides with L₇₅-R₈₄ peptide moiety (see Figs 5, 7, 8 and 9). Due to the lack of a trypsin consensus motif at Y₇₄-L₇₅, the reason for the N-terminal cleavage is not clear. ^bGlycopeptides with S₄₅-K₆₇ peptide moiety detected after reduction and alkylation.

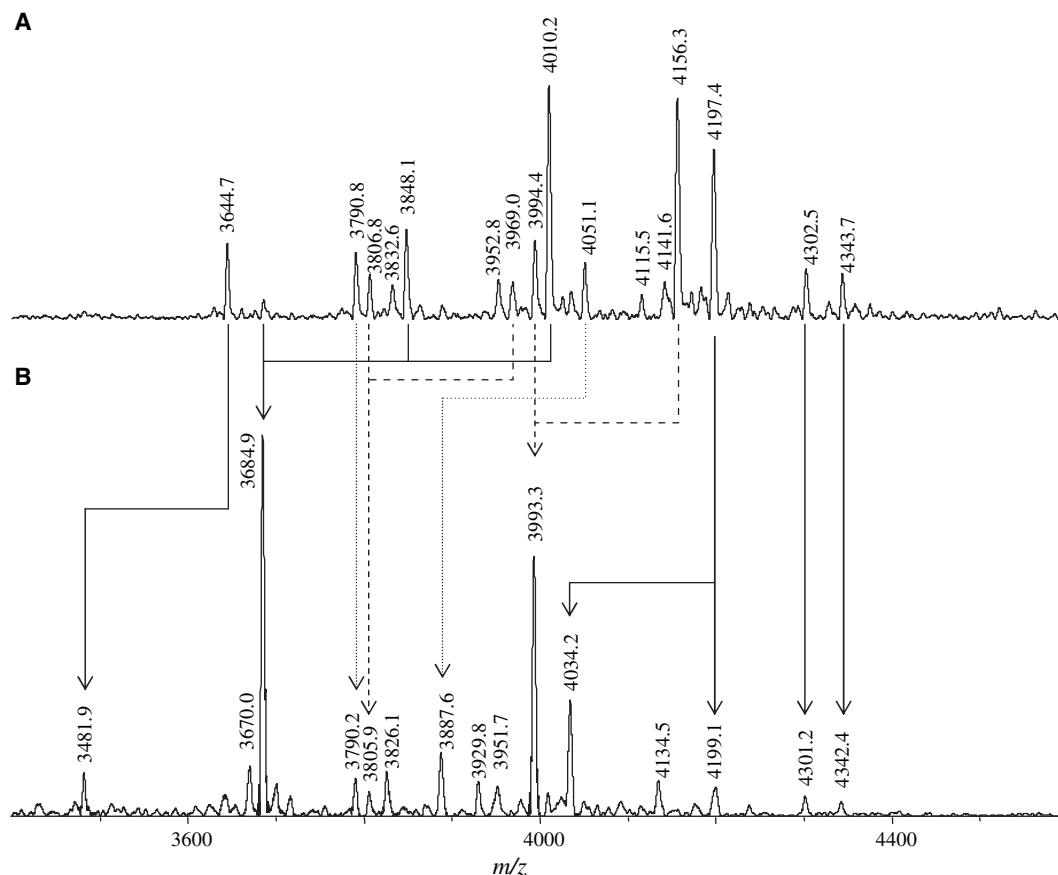


Fig. 4. β -Galactosidase treatment of glycopeptides. Glycopeptide fraction 42 obtained by RP-HPLC fractionation of the tryptic digest of the interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1) was analysed by MALDI-TOF-MS before (A) and after (B) treatment with β -galactosidase. Related peaks are connected by arrows, indicating the loss of two, one or no hexoses.

and fucose), and m/z 1693 (protonated peptide with *N*-acetylglucosamine and two fucoses) indicated a peptide mass of 1198 Da $[M+H]^+$ and a glycan moiety of composition $H_5N_4F_3$. For further analysis of peptide sequence and glycan attachment sites, glycopeptides of fractions 23–25 were enzymatically deglycosylated by treatment with PNGase A. The resulting deglycosylated peptide was identified by nano-LC-MS/MS as L75–R84, covering the glycosylation site, N80. Enzymatic glycan removal converted N80 to aspartate (D), which confirmed the glycan attachment site (Fig. 6A,B; discussed below). While the C terminus of the L75–R84 glycopeptides is in accordance with cleavage by trypsin, the N-terminal cleavage site lacks a preceding R or K residue, which is required for tryptin activity. The cleavage between Y74 and L75 might have already been present in part of the natural IPSE molecules, or might have occurred during purification. Alternatively, this cleavage might have been the result of the action of trypsin at a nonconsensus site.

In order to obtain information on antenna composition and core substitution, glycopeptides were analyzed by nano-LC-MS/MS. The fragmentation data of four of these glycopeptides are shown in Figs 7–10. The fragmentation data of the other glycopeptides are summarized in Table 1. The MS/MS data revealed difucosylation of the innermost *N*-acetylglucosamine for most of the glycopeptides. Glycopeptide L75–R84 with the *N*-glycan $H_3N_2F_2$ was interpreted as a trimannosyl *N*-glycan with two fucoses attached to the innermost *N*-acetylglucosamine selecting an $[M+2H]^{2+}$ precursor (Fig. 7). The signal at m/z 674 was considered to arise from fucose rearrangement of the protonated species. In this context it has to be noted that the fragmentation of protonated, fucose-containing glycans is known to result in migration of the fucose residues, and this undesired effect complicates the interpretation of MS/MS spectra ([28] and references cited therein). The signal at m/z 1365 is believed to be related to the prominent signal at m/z

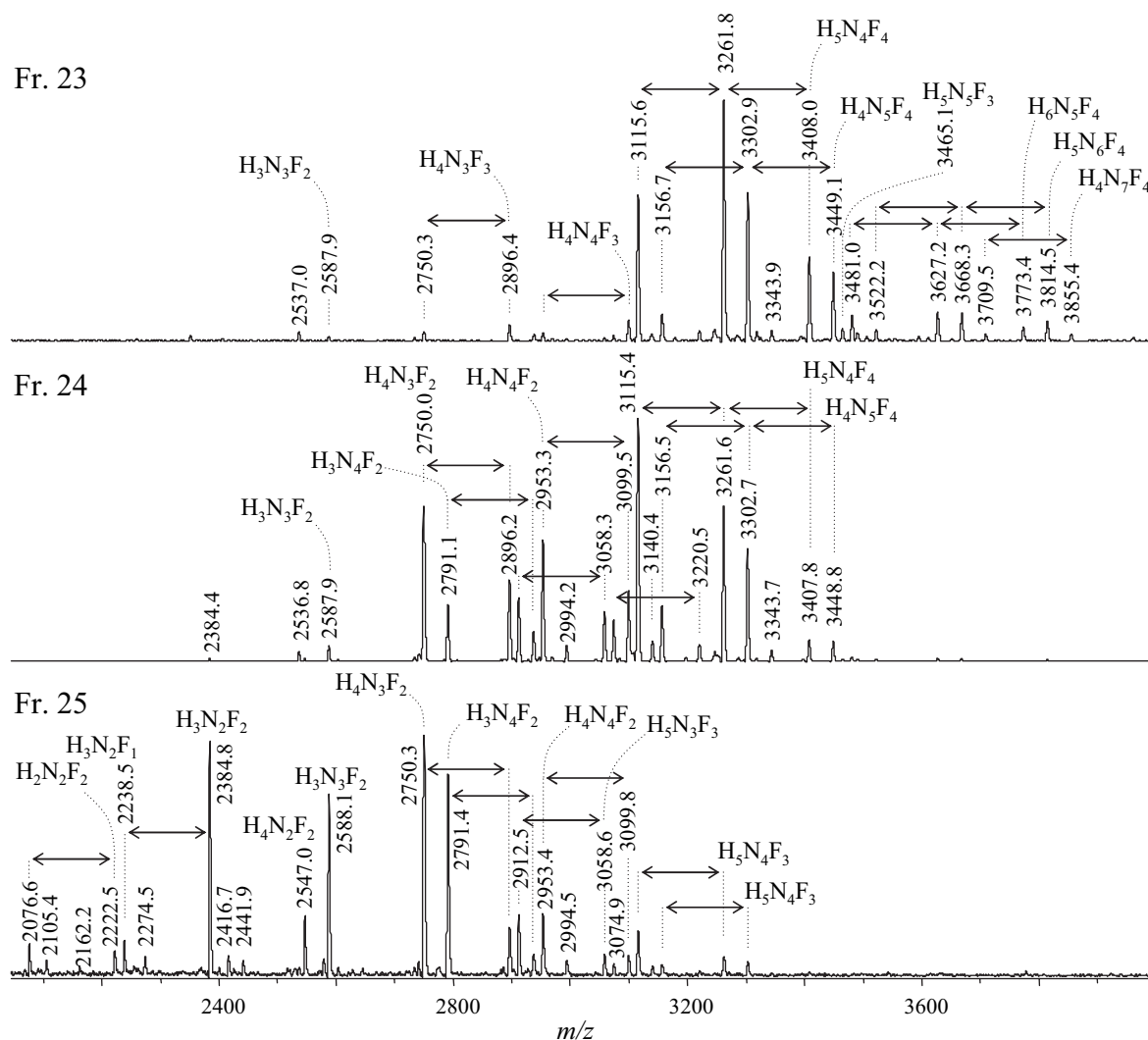


Fig. 5. MALDI-TOF-MS of glycopeptides L75–R84. On HPLC fractionation of the tryptic digest of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1), these glycopeptides were detected in fractions 23–25. The signals are labeled with average masses. Compositions of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N), and fucose (F). Differences in fucose content are indicated by double-headed arrows.

1401 (peptide with one *N*-acetylglucosamine) and might arise by the elimination of two water molecules. Likewise, the signal at m/z 1511 corresponds to the species at m/z 1547 lacking two water molecules.

Glycopeptide L75–R84 with the N-glycan, $H_5N_4F_2$, exhibited two LacNAc antennae and core-difucosylation (Table 1), as confirmed by the loss of two galactoses on β -galactosidase treatment (Fig. 4). Glycopeptide L75–R84, with the glycan $H_5N_4F_4$, was found to contain two monofucosylated antennae (Fig. 8), which, based on data reported in the literature [5,29,30], are interpreted as Lewis X units, and this is consistent with its resistance to β -galactosidase treatment (Table 1 and Fig. 4). The fragment ion at m/z

658 in Fig. 8 was interpreted as a Lewis X unit with an additional fucose residue, and probably originates from a fucose migration process associated with glycopeptide fragmentation (see above). This interpretation is supported by the complete galactosidase resistance of glycopeptides with the glycan $H_5N_4F_4$, which is in accordance with a monofucosylation of both antennae and difucosylation of the core. Besides the signal at m/z 658, the double-charged ion at m/z 1521 is generated via a fucose-rearrangement process: this ion exhibits the loss of a LacNAc antenna with retention of all fucose residues. Fragment ions at m/z 658 and 699 (protonated LacdiNAc with two fucoses) were observed also for several other glycopeptide species

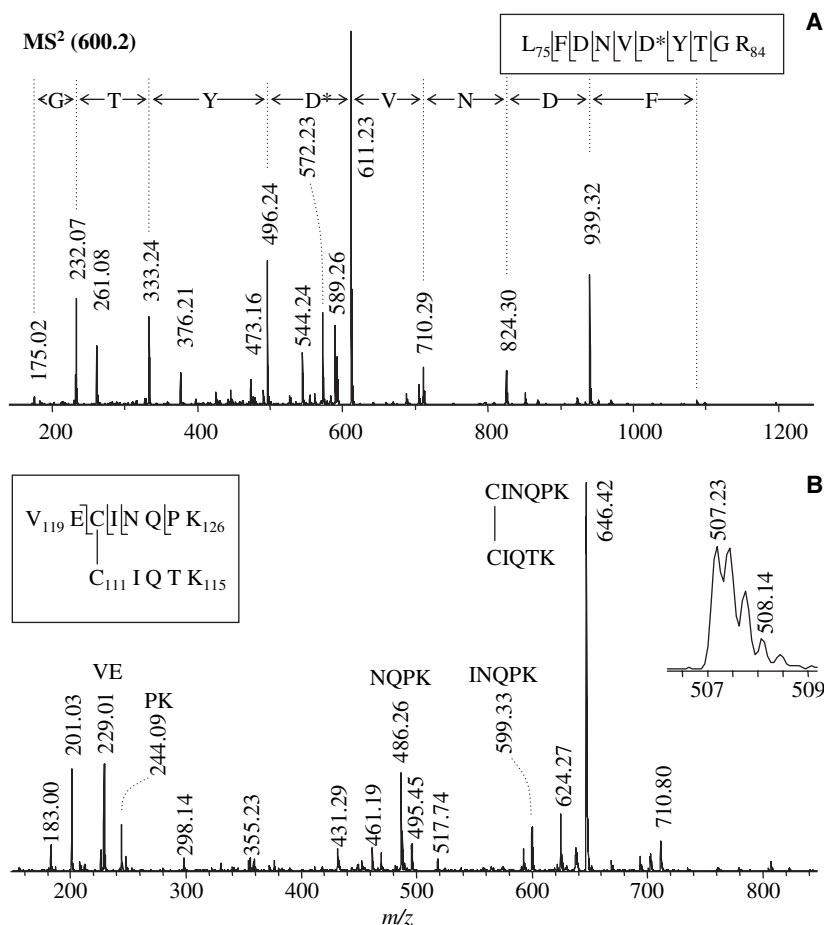


Fig. 6. Analysis of glycan attachment site and disulfide linkage. (A) Glycopeptide fractions 23–25 (Fig. 5) were treated with PNGase A and the deglycosylated peptide obtained (L75–R84) was sequenced by nano-LC-MS/MS. The aspartate generated by enzymatic release of the glycan moiety is labeled with an asterisk (*). (B) Nano-LC-MS (inset) and MS/MS of a disulfide-linked dipeptide of the tryptic digest of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1).

(Table 1). Yet, β -galactosidase treatment revealed nonfucosylated and monofucosylated antennae throughout, and the above-mentioned ions suggesting difucosylated antenna species were therefore interpreted as rearrangement products.

Glycopeptides L75–R84 with glycans of composition $H_4N_5F_3$ carried one LacNAc and one LDN antenna, which were found to be partially fucosylated (Fig. 9). They were partially resistant to β -galactosidase treatment ($\approx 30\%$ of the signal remained at m/z 4199, Fig. 4B). Seventy per cent of these glycans exhibited a loss of one hexose, resulting in a signal at m/z 4034 (Fig. 4B). The partial removal of one galactose was not the result of inefficiency of the enzyme, as the structurally similar glycopeptide, L75–R84, with an $H_4N_5F_2$ N-glycan, exhibited a complete loss of its terminal galactose moiety upon β -galactosidase treatment under the same conditions. Rather, the incomplete hexose removal seemed caused by the heterogeneity in fucose positions: the majority of the glycopeptides contain a LacNAc antenna and a fucosylated LacdiNAc antenna, while the others contained a Lewis X antenna

in combination with an LDN antenna and were thus completely resistant to β -galactosidase treatment. The mass spectrometric data do not allow differentiation between F-LDN and LDN-F motifs, and both elements have been shown to be present on many *S. mansoni* egg glycoproteins [5]. In analogy with the adult worms, which express LDN-F rather than F-LDN on N-glycans ([31], M. Wührer, unpublished results), the interpretation of fucosylated LacdiNAc of IPSE/ α -1 as predominantly LDN-F seems favourable.

The fragmentation data on the disulfide-linked dipeptide I85–K99/S45–K67 carrying the N-glycan $H_5N_4F_3$ at N-glycosylation site N64 was obtained from the total tryptic digest of IPSE/ α -1 and indicated core-difucosylation as well as a LacNAc and a Lewis X antenna (Fig. 10). After reduction and alkylation, glycan $H_5N_4F_3$ was detected on the carbamidomethylated peptide, S45–K67, by nano-LC-MS (m/z 1131.6 $[M + 3H + Na]^{4+}$; see Table 1). The mass shift observed on reduction and alkylation thus corroborated the identity of the peptide moiety of this glycopeptide.

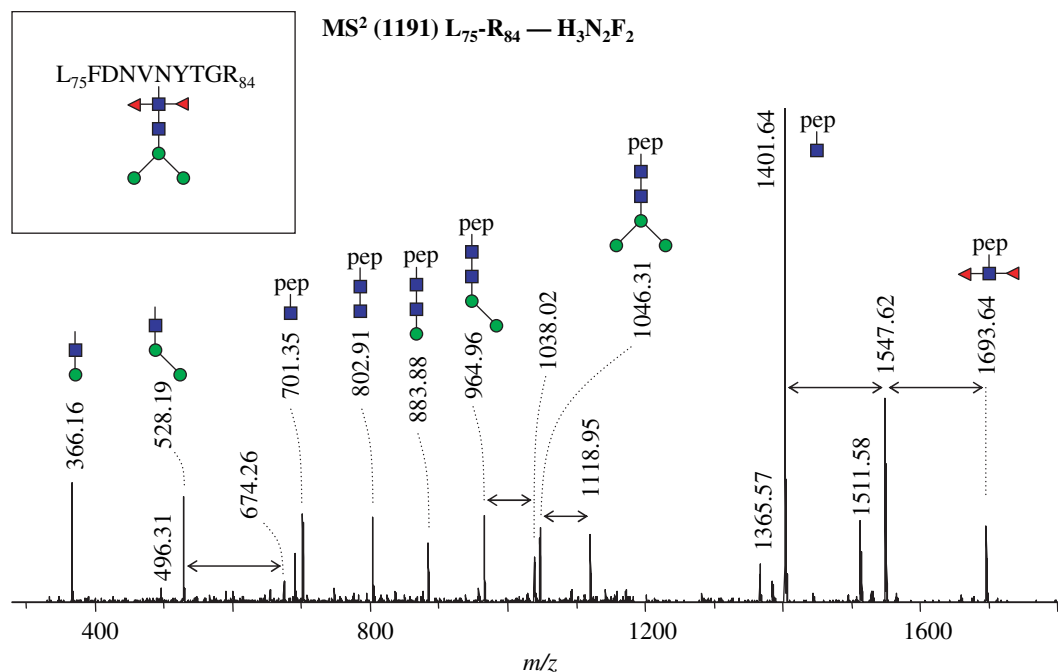


Fig. 7. MS/MS of peptide L75–R84 carrying H₃N₂F₂ N-glycans. The MS/MS spectrum was acquired by nano-LC-MS/MS of HPLC fraction 23 selecting an [M+3H]³⁺ precursor. Red triangle, fucose; blue square, N-acetylglucosamine; green circle, mannose.

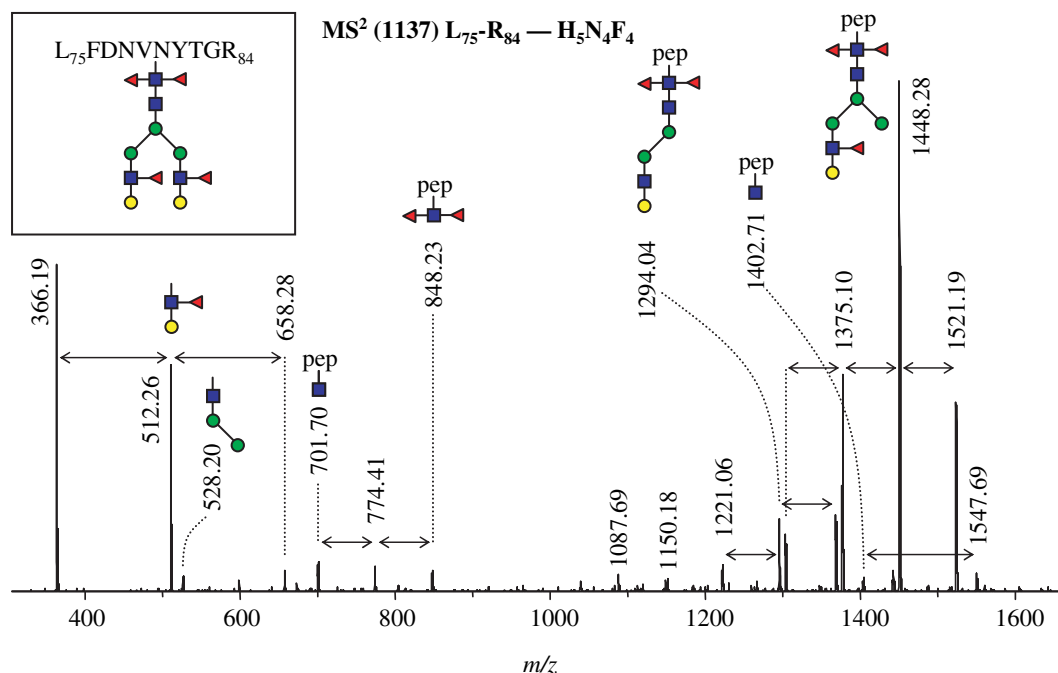


Fig. 8. MS/MS of peptide L75–R84 carrying H₅N₄F₄ N-glycans. The MS/MS spectrum was acquired by nano-LC-MS/MS of HPLC fraction 25 selecting an [M+3H]³⁺ precursor. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

In summary, the direct analysis of tryptic glycopeptides by MALDI-TOF-MS and nano-LC-MS/MS, or in combination with β -galactosidase treatment,

PNGase A treatment, and reduction/alkylation, revealed similar populations of N-glycans for the two consensus N-glycosylation sites N80 and N64. An

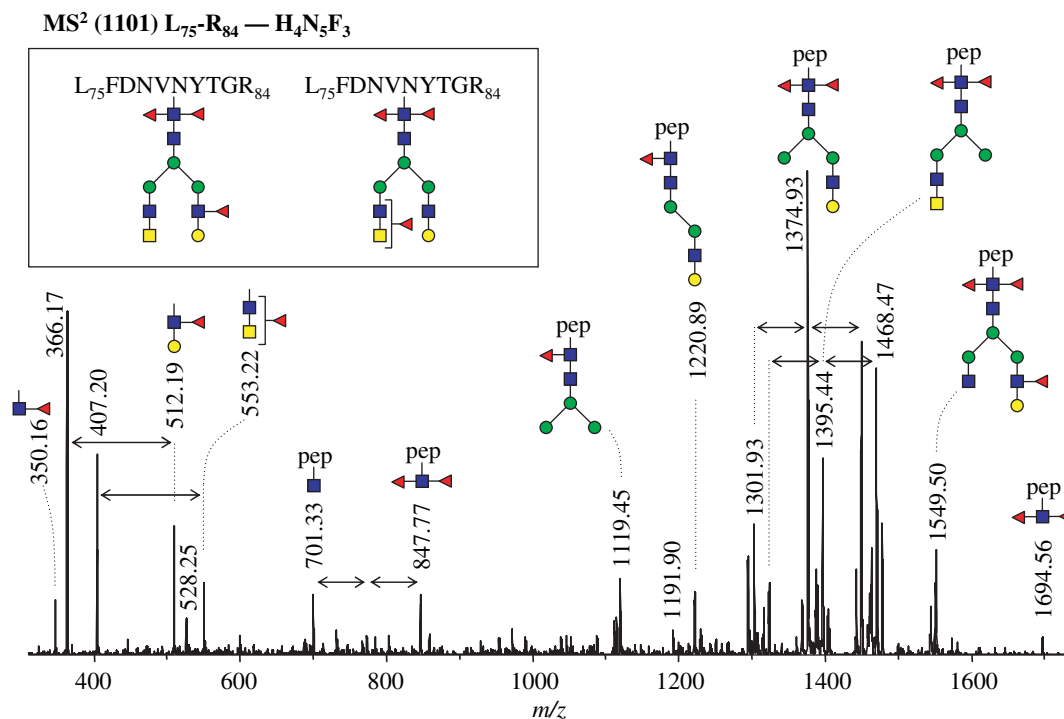


Fig. 9. MS/MS of peptide L₇₅–R₈₄ carrying H₄N₅F₃ N-glycan. The MS/MS spectrum was acquired by nano-LC-MS/MS of HPLC fraction 24 selecting an [M+3H]³⁺ precursor. Red triangle, fucose; yellow circle, galactose; yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; green circle, mannose.

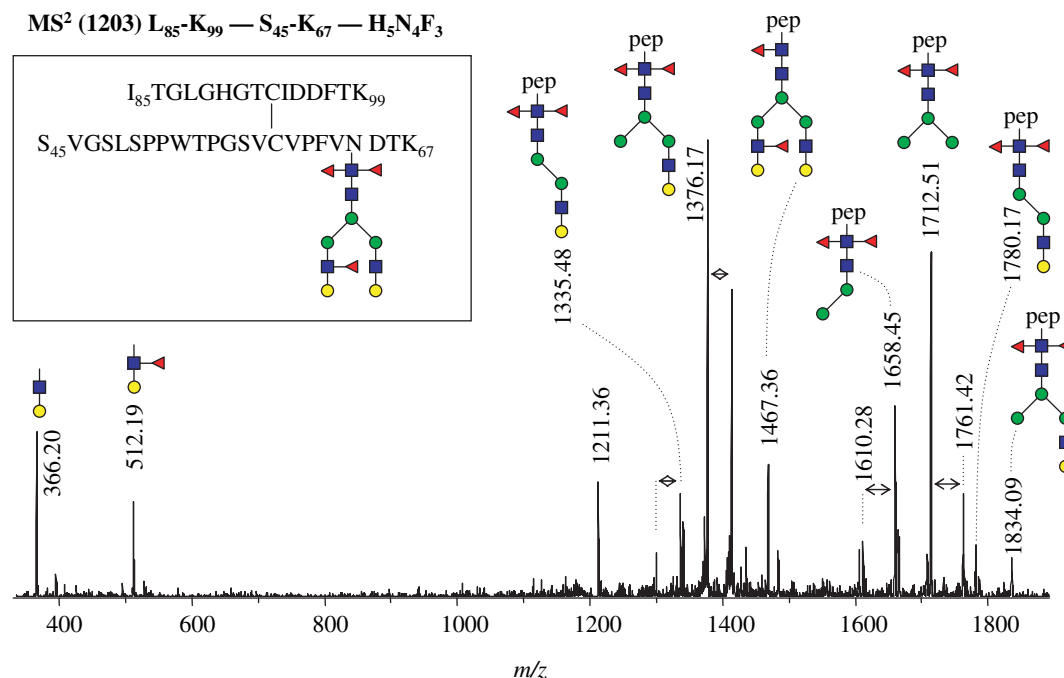


Fig. 10. MS/MS of the disulfide-linked dipeptide L₈₅–K₉₉/S₄₅–K₆₇ carrying H₅N₄F₃ N-glycans selecting an [M+3H]³⁺ precursor. The MS/MS spectrum was acquired by nano-LC-MS/MS of the total tryptic digest of interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1). Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

overview of the N-glycosylation of IPSE/ α -1 is given in Fig. 11. Most of the N-glycans exhibit a difucosylated innermost *N*-acetylglucosamine. The majority of the N-glycans contain two LacNAc antennae, which may each be fucosylated, resulting in one or two Lewis X units (N1 in Fig. 11). The second type of N-glycan, in terms of abundance, exhibits one LacNAc and one LacdiNAc antenna. Again, both of these antennae may be monofucosylated, resulting in Lewis X and fucosylated LacdiNAc, respectively (N2 in Fig. 11). Third in abundance are monoantennary N-glycans with a LacNAc or a Lewis X terminal unit (N3). As shown in Fig. 11, various other, less-abundant combinations of up to three LacNAc and/or LDN antennae, with or without fucosylation, occur.

Other structural aspects of IPSE/ α -1, revealed by MS

Besides the analysis of the N-glycosylated glycopeptides, the chosen approach provided additional information on the structural features of IPSE/ α -1. MALDI-TOF-MS revealed the mass of the intact antigen (Fig. 2A), and in-source decay resulted in a series of ions in the mass range between m/z 1500 and m/z 4500 (Fig. 2B), which were identified as N-terminal protein fragments. These fragments confirmed D21 as the N terminus of IPSE/ α -1 (Fig. 1). The fact that N-terminal fragments were observed, indicated that C23 and C26 formed an intrachain disulfide bridge, as any other disulfide bond would have prevented these fragments.

A second disulfide bridge was detected between C59 and C93 during the analysis of the glycopeptides (Figs 3 and 10 and Table 1). A third disulfide bridge was registered by LC-MS/MS of the total tryptic digest: the automatic MS/MS of an intense, triple-charged species at m/z 507 identified this as a C111–K115/V119–K126 dipeptide (Fig. 6B), thus establishing a disulfide bridge between C111 and C121. While C23 and C26 form an intrachain disulfide bridge, the disulfide bonds between C59 and C93, as well as between C111 and C121, could be either within one IPSE/ α -1 monomer or between the two polypeptide chains of the IPSE/ α -1 dimer. No peptides covering C132 and the C terminus of IPSE/ α -1 were detected in LC-MS/MS, either with or without reduction/alkylation. Based on the current data, C132 might be involved in an interchain disulfide bond of the IPSE/ α -1 dimer [24]. Nano-LC-MS(/MS) of the total tryptic digests, without reduction/alkylation, additionally revealed the peptides D20–R36 (with an internal disulfide bridge), L27–R36, G37–K44, E69–F76, S100–R110 and the disulfide-linked dipeptides C111–K115/V119–K126 (Fig. 6B) and C111–K115/D116–K126. After reduction/alkylation, the latter two dipeptides disappeared, and V119–K126 and I85–K99 were registered with alkylated cysteine moieties. Together with the above-mentioned glycopeptides, these peptides result in an almost complete coverage of the IPSE/ α -1 sequence. None of these peptides were detected with post-translational modifications, such as O-glycosylation, indicating that N-glycosylation is the only modification on IPSE/ α -1.

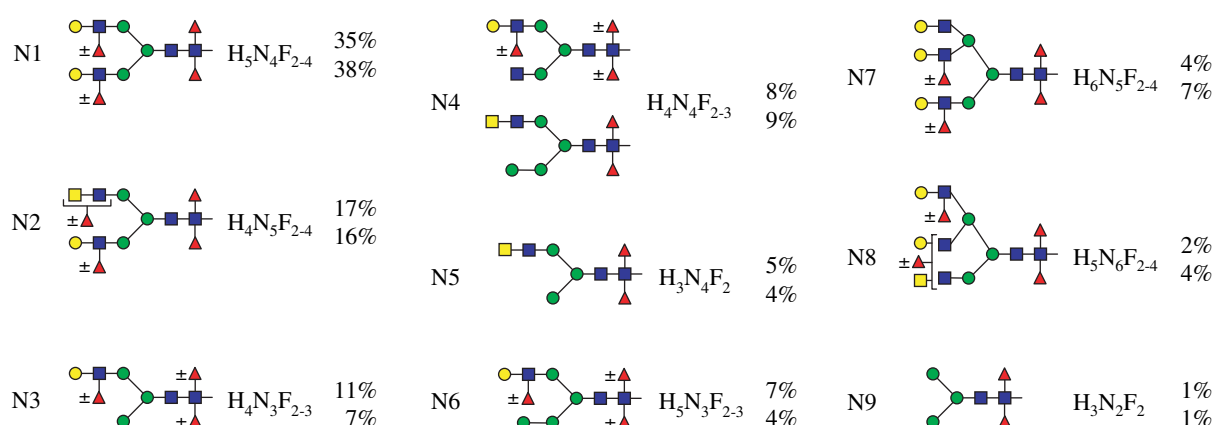


Fig. 11. Schematic representation of the N-glycans detected on interleukin-4-inducing factor from schistosome eggs (IPSE/ α -1). N1 to N9 refers to Table 1. The relative abundance of each glycan group is given for site N80 (upper numbers) and N64 (lower numbers). Numbers are based on glycopeptide signal intensities in MALDI-TOF-MS. Intensities of species N1 to N9 sum up to 90%, leaving an estimated 10% for unassigned minor species. Red triangle, fucose; yellow circle, galactose; yellow square, *N*-acetylgalactosamine; blue square, *N*-acetylglucosamine; green circle, mannose.

N-glycosylation site occupancy

While glycosylation site N64 seemed to be fully occupied (unglycosylated dipeptide L85–K99/S45–K67 was not detected), the peptide E69–R84 was detected both with and without N-glycosylation, indicating a partial occupation of site N80. Thus, four N-glycans may be present per IPSE/ α -1 disulfide-linked dimer. Taking into account the average mass of one N-glycan of ≈ 2 kDa and the MALDI-TOF-MS data for intact IPSE/ α -1, this means that the majority of IPSE/ α -1 (estimated 70% based on Fig. 2A) contains four N-glycans (two polypeptides with two N-glycans each) and accounts for the major signal at 35 kDa. (Fig. 2A). The shoulder at 33 kDa is interpreted as IPSE/ α -1 with three N-glycans (one polypeptide chain with two N-glycans and one polypeptide chain with one N-glycan) and would account for an estimated 30% of total IPSE/ α -1.

Discussion

Schistosome-derived glycans, including those that contain the Lewis X motif, fucosylated-LDN termini, or α 3-fucosylated core-chitobiose are of interest as potential immunomodulatory molecules [20,32,33], and as possible targets for immune intervention of schistosomiasis [34]. As no structural data on the protein-specific glycosylation of any particular schistosome egg protein have been reported to date, it is not known which proteins express these immunogenic glycan motifs. Moreover, several well-known SEAs, including omega-1, IPSE/ α -1 [26] and k-5 [35], are glycoproteins, but structural information on these proteins at the glycan level is still completely lacking. This prompted us to study the post-translational modifications of IPSE/ α -1, one of the major secretory *S. mansoni* egg glycoprotein antigens [24–27,36], as a first step towards the detailed characterization of the schistosome egg glycoproteome. Mass spectrometric analysis of IPSE/ α -1 at the (glyco-)peptide level revealed that the protein carries glycans at two N-glycosylation sites, of which one is not always occupied. The MALDI-TOF-MS profile (Fig. 2A) suggests that $\approx 70\%$ of the IPSE/ α -1 dimers carry four N-glycan chains and the rest carry three N-glycan chains. This is in full agreement with the initial observations that α -1 occurs in two forms, both consisting of two glycoprotein subunits [26]. In that study, the major form was found to be a homodimer of two subunits of identical mass, and the minor form appeared to be a heterodimer of two subunits which have a mass difference attributable to approximately one N-glycan

[26]. The current observations indicate that the formerly postulated heterodimer is formed by two different glycoforms of the IPSE/ α -1 monomer, one form with both N-glycosylation sites occupied, and one which carries only a single N-glycan. In the homodimer of higher molecular mass, both sites on both monomers carry N-glycans.

As the Lewis X motif can be present at both N-glycosylation sites with an estimated 0.6 Lewis X units per glycan chain (Table 2), an average of ≈ 2.2 Lewis X units are present on each IPSE/ α -1 dimer, with most glycans having an (α 1-3) and/or (α 1-6)-(di)fucosylated core (see Table 1, Fig. 11 for an overview). Although it has been well established that a subset of schistosome egg glycans contains Lewis X [5,15], this is the first time that Lewis X glycans, or in fact any type of defined glycans, have been assigned to a particular carrier protein from schistosome eggs.

The finding that IPSE/ α -1 N-glycans carry Lewis X motifs as well as typical helminth/plant antigenic core- α 3-fucosylation is highly significant because several publications have reported that Lewis X, and possibly other fucosylated glycans, are involved in the immunomodulatory activities of schistosome egg-derived glycoprotein preparations [32,33,37]. In schistosomiasis, the early phase of infection is characterized by Th1 immune responses that progress to Th2 upon egg laying. The question of how schistosomes and other helminths skew immune responses towards Th2 has received much attention and recent studies have identified several molecular patterns that induce strong Th2 polarization of immune responses. It has been reported that core α 3-fucose-containing N-glycans within the soluble egg antigen preparations induce Th2 polarization when tested *in vivo* [33]. It is not known, however, whether these N-glycans also carried other potentially immunostimulatory glycan elements such as observed in the case of IPSE/ α -1 (Fig. 11). Lewis X, in the context of LNFPIII-conjugates, triggers Th2-type responses, including production of IL-4 and IL-10 following intranasal immunization [18,32]. Stimulation of peripheral blood mononuclear cells (PBMCs), from *S. mansoni*-infected individuals, with LNFPIII conjugated to human serum albumin, induced the production of IL-10 [38]. Innate immune signalling of Lewis X neoglycoconjugates occurs through Toll-like receptor (TLR4) stimulation [39]. In addition, it has been reported that DC-SIGN, which binds to Lewis X and various other pathogen and nonpathogen-derived glycans, enables the uptake of Lewis X antigens [40]. It should be noted that multivalency of Lewis X may be an important factor in the observed immunomodulatory effects. The LNFPIII-HSA neoglycoconjugate induces

Table 2. Expression of Lewis X on interleukin-4-inducing factor from schistosome eggs (IPSE/ α 1). Based on the peak intensities in MALDI-TOF-MS and the structural characteristics determined for glycan species N1–9, the occurrence of terminal Lewis X motifs is estimated. For site N₈₀ substituted with the glycan group N1, for example, the glycopeptide detected at *m/z* 4301 (Fig. 3A) corresponds to an estimated 4% of the total glycopeptide species of this site and exhibits two Lewis X units, whilst the dominant signal at *m/z* 4155 corresponds to glycan species with 18% relative abundance exhibiting one Lewis X unit. Expression of Lewis X for the minor glycan species (others) was expected to be in line with the results obtained for N1–N9. In conclusion, glycans on both sites each carry ≈ 0.6 Lewis X units on average. Le^X, Lewis X.

Glycans	Relative abundance (%)	N-glycosylation site N ₈₀ Expression of Le ^X (%)			Le ^X units	Relative abundance (%)	N-glycosylation site N ₆₄ Expression of Le ^X (%)			Le ^X units
		0 Le ^X	1 Le ^X	2 Le ^X			0 Le ^X	1 Le ^X	2 Le ^X	
N1	35	13	18	4	0.26	38	15	16	7	0.30
N2	17	9	8	0	0.08	16	8	8	–	0.08
N3	11	5	6	0	0.06	7	3	4	–	0.04
N4	8	4	4	0	0.04	9	5	4	–	0.04
N5	5	5	0	0	–	4	4	–	–	–
N6	7	2	5	0	0.05	4	2	2	–	0.02
N7	4	1	2	1	0.04	7	3	3	1	0.05
N8	2	1	1	0	0.01	4	2	2	0	0.02
N9	1	1	0	0	–	1	1	0	0	–
others	10	4	5	1	0.07	10	5	4	1	0.06
Σ	100	45	49	6	0.61	100	48	43	9	0.61

IL-10 production in PBMCs, whereas pretreatment of PBMCs with free LNFPIII attenuates SEA-induced IL-10 production [38]. This indicates that multivalent Lewis X presentation has a stimulatory affect, with the unconjugated monomer acting as an antagonist. In this respect, it is relevant that a large proportion of IPSE/ α 1 carries two or more Lewis X elements.

IPSE/ α 1 was identified as the bioactive component in *S. mansoni* egg extract, leading to the activation of basophils and to the expression of IL-13 and IL-4, the latter cytokine being a key player in Th2 biasing. Glycosylation of IPSE/ α 1 is not required for this activity, as nonglycosylated recombinant IPSE/ α 1 produced in *Escherichia coli* displays similar activity as the purified glycoprotein [24]. This would mean that the basophil-associated IL-4-inducing capacity of IPSE/ α 1 is purely protein dependent. In view of the reported activities of Lewis X neo-glycoconjugates, which are capable of acting as an adjuvant by skewing the immune system towards Th2 responses in a protein-independent manner [18], it would be very interesting, however, to evaluate whether other immune reactions to IPSE/ α 1 would be dependent on, or modulated by, its natural Lewis X glycans. Possibly, the IPSE/ α 1 protein and the Lewis X glycans expressed by IPSE/ α 1 can each affect the host's immune response through different pathways.

The presence of Lewis X, LDN, fucosylated LDN (F-LDN/LDN-F) and core α 3-fucose (Fig. 11) implies that IPSE/ α 1 is among the proteins that

are targeted, on the basis of their glycosylation, by the host's humoral immune system. Antibody responses have been measured to each of these motifs in schistosomiasis in various natural and experimental settings [4,12–14]. The (α 1-3)/(α 1-6) difucosylated core-chitobiose has previously been observed in *S. mansoni* (trematode) egg glycans [15] and also in the nematode *Haemonchus contortus* [41]. Core α 3-fucose forms an important epitope for immunoglobulin E (IgE) in helminth infections [42] but also in allergic reactions to plant glycans [43]. Whether glycans such as those found on IPSE/ α 1 might be potential schistosomiasis vaccine targets is still an issue of debate [4,34].

Notably, LDN forms a pattern for galectin-3-mediated immune recognition [44]. It would be interesting to determine whether the subset of N-glycans that contain LDN plays a role in the uptake of IPSE/ α 1 by phagocytic host cells.

Although it is not clear at present which other egg-derived glycoproteins express Lewis X, a western blotting study using an anti-Lewis X mAb indicated that only a particular subset of all egg glycoproteins is reactive with this mAb [5]. Moreover, compared with the overall glycosylation of egg proteins, IPSE/ α 1 expresses only a small subset of all N-glycans previously determined (Table 1) [15]. IPSE/ α 1 has been localized to a particular subegg shell compartment [27] and it may share its glycosylation properties with other proteins synthesized and transported via the

same pathway, and thus encountering the same glycosylation machinery.

Experimental procedures

Purification of IPSE/ α -1

IPSE/ α -1 was initially purified from 15 mg of *S. mansoni* SEA by ion-exchange chromatography on a 5 mL CM-Sepharose column (Amersham Biosciences, Bucks., UK), as previously described [26], followed by immunoaffinity chromatography using polyclonal rabbit anti-IPSE/ α -1 covalently coupled to Sepharose-4B. The bound IPSE/ α -1 fraction was eluted with 0.58% acetic acid/0.5 M NaCl, adjusted to pH 7.2 by the addition of diluted NaOH and concentrated to 1 mL using a YM10 pore exclusion membrane (Millipore, Watford, UK) in a pressurized stirred concentration cell (model 8010; Millipore), followed by three additions of 5 mL NaCl/P_i, each time reconcentrating to 1 mL, to give a final amount of ≈ 25 μ g of IPSE/ α -1, measured using a BCA Protein Assay Kit (Perbio, Cramlington, UK).

Sample preparation

For trypsin treatment, ≈ 10 μ g of IPSE/ α -1 (400 μ L; 1 volume) was incubated with 1 μ g of trypsin (Promega, Leiden, the Netherlands) in 25 μ L of 50 mM ammonium bicarbonate at 37 °C overnight. The sample was stored at -20 °C and used for MALDI-TOF-MS, HPLC fractionation and nano-LC-MS/MS. For reduction and alkylation, the sample was mixed with 0.05 volumes of 200 mM dithiothreitol, incubated for 45 min at 56 °C, after which 0.2 volumes of 200 mM iodoacetamide were added and the sample incubated for 30 min in the dark at room temperature. For β -galactosidase treatment, glycopeptides of HPLC fractions 41 and 42 were treated with β -galactosidase from jack beans (50 mU; Seikagaku, Tokyo, Japan) in 100 mM sodium acetate buffer, pH 5.0, for 1 h at 37 °C.

RP-HPLC fractionation

The IPSE/ α -1 tryptic digest was fractionated on a Supersphere 100 C₁₈ endcapped column (4 μ m, 250 $\times$ 4 mm; Merck, Darmstadt, Germany) at a flow rate of 1 mL $\cdot$ min⁻¹. The column was equilibrated with solvent A (0.1% trifluoroacetic acid). After injection, a gradient within 4 min to 20% solvent B (80% acetonitrile, 0.1% trifluoroacetic acid), and within an additional 36 min to 70% solvent B was applied. The UV absorbance was measured (at 205 nm, 215 nm and 280 nm), and fractions of 0.5 mL were collected. Fractions were dried in a vacuum centrifuge and dissolved in 50 μ L of water.

MALDI-TOF(/TOF)-MS

Intact IPSE/ α -1, total tryptic digest of IPSE/ α -1, as well as glycopeptides after β -galactosidase treatment, were purified by Zip-Tip_{C18} following the manufacturer's instructions (Millipore, Billerica, MA, USA), eluted with 50% acetonitrile, 0.1% trifluoroacetic acid onto the MALDI target plate, and mixed with 1 μ L of matrix solution (6-aza-2-thiothymine, 5 mg $\cdot$ mL⁻¹ in water; Sigma, Swijndrecht, The Netherlands). RP-HPLC fractions were directly mixed with matrix solution on the target plate. Sample spots were dried under a stream of warm air. MALDI-TOF(/TOF)-MS data were obtained using an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a LIFT-MS/MS facility. Spectra were acquired in the positive-ion linear mode (intact IPSE/ α -1) and positive-ion reflectron mode (glycopeptides and peptides). For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage.

Nano-HPLC ESI-ion trap-MS

(Glyco-)peptide samples were applied to a reverse-phase column (PepMap, 3 μ m, 75 μ m $\times$ 100 mm; Dionex/LC Packings, Amsterdam, the Netherlands) using an Ultimate nano-LC system, a Famos autosampler, and a Switchos trap-column system (Dionex/LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 200 nL $\cdot$ min⁻¹. After injection of the sample, elution conditions were switched to 10% solvent B (95% acetonitrile, 0.1% formic acid), followed by a gradient to 60% B in 45 min and a subsequent isocratic elution of 10 min. The eluate was monitored by absorption at 215 nm.

The LC column was coupled to an Esquire HCT ESI-IT-MS (Bruker) equipped with an online nanospray source operating in the positive-ion mode. For electrospray (1100–1250 V), capillaries (360 μ m OD, 20 μ m ID with a 10 μ m opening) from New Objective (Cambridge, MA, USA) were used. The solvent was evaporated at 175 °C employing a nitrogen stream of 7 L $\cdot$ min⁻¹. Ions from m/z 200 to m/z 2500 were registered. When operated in the auto MS/MS mode, each MS scan was followed by the acquisition of MS/MS spectra of up to three of the most abundant ions in the MS spectrum.

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